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(71) Applicant *(for all designated States except US)*: ELI LILLY AND COMPANY [US/US]; Lilly Corporate Center, Indianapolis, IN 46285 (US).

(71)(72) Applicants and Inventors: VASUDEVAN, Venkatraghavan [US/US]; 1016 Saratoga Circle, Indianapolis, IN 46280 (US). JONES, Thomas, Warren [US/US]; 9547 Timberline Drive, Indianapolis, IN 46256 (US). RODRIGUEZ, Michael, John [US/US]; 7649 Gordonshire Court, Indianapolis, IN 46278 (US). SWEETANA, Stephanie, Ann [US/US]; 4112 Saratoga Drive, Bloomington, IN 47408 (US).

(74) Agents: MUSSER, Arlene, K. et al.; Eli Lilly And Company, Lilly Corporate Center, Indianapolis, IN 46285 (US).

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(57) Abstract

Methods and compositions for treating fungal infections that include formulations of a pseudomycin or related antifungal agent and a cyclodextrin are described. The compositions are particularly useful in pharmaceutical applications to reduce adverse effects.

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PSEUDOMYCIN ANTIFUNGAL COMPOSITIONS AND METHODS FOR THEIR USE

FIELD OF THE INVENTION

The present invention relates to methods and compositions for treating fungal infections that include formulations of a pseudomycin or a lipodepsidecapeptide and a cyclodextrin, in particular, hydroxypropyl-β-cyclodextrin or sulfobutylether-β-cyclodextrin.

BACKGROUND

Fungal infections are a significant cause of disease, degradation of quality of life, and mortality among humans, particularly for immune compromised patients. The incidence in fungal infections in humans has increased greatly in the past 20 years. This is in part due to increased numbers of people with immune systems weakened or devastated by organ transplants, cancer chemotherapy, AIDS, age, and other similar disorders or conditions. Such patients are prone to attack by fungal pathogens that are prevalent throughout the population but are kept in check by a functioning immune system. These pathogens are difficult to control because some existing antifungal agents are either highly toxic or only inhibit fungal activity. For example, the polyenes are fungicidal but toxic; whereas, the azoles are much less toxic but only fungistatic. More importantly, there have been recent reports of azole and polyene resistant strains of *Candida* which severely limits therapy options against such strains.

One class of new antifungal agents, the pseudomycins, shows great promise for treating fungal infections in a variety of patients. Pseudomycins are natural products derived from isolates of *Pseudomonas syringae*. *P. syringae* is a large family of plant bacteria that have been the source of several bioactive substances, such as bacitracin and the syringomycins. Natural strains and transposon generated mutants of *P. syringae* produce compounds with antifungal activity. A transposon generated regulatory mutant of the wild type strain of *P. syringae* 174, known as MSU 16H (ATCC 67028), produces several pseudomycins. For example, pseudomycins A, B, C and C' have each been

isolated and purified and their structures have been characterized by methods including amino acid sequencing, NMR, and mass spectrometry and have been shown to possess wide spectrum antifungal activity, including activity against important fungal pathogens in both humans and plants. See, e.g. Ballio et al., "Novel bioactive lipodepsipeptides from *Pseudomonas syringae*: the pseudomycins," FEBS Lett. **355**, 96-100 (1994) and U.S. Patent No. 5,576,298. The pseudomycins, the syringomycins, the syringotoxins, and the syringostatins represent structurally distinct families of antifungal compounds.

As yet, formulations of pseudomycins and related antifungal agents from *P. syringae* that are effective for antifungal therapy have not been developed. Typically injection of a pseudomycin is accompanied by unacceptable adverse side effects such as irritation at the injection site. There remains a need for a composition of a pseudomycin or a related antifungal agent that can be used for treating fungal infections without the adverse side effects observed in certain formulations of pseudomycins.

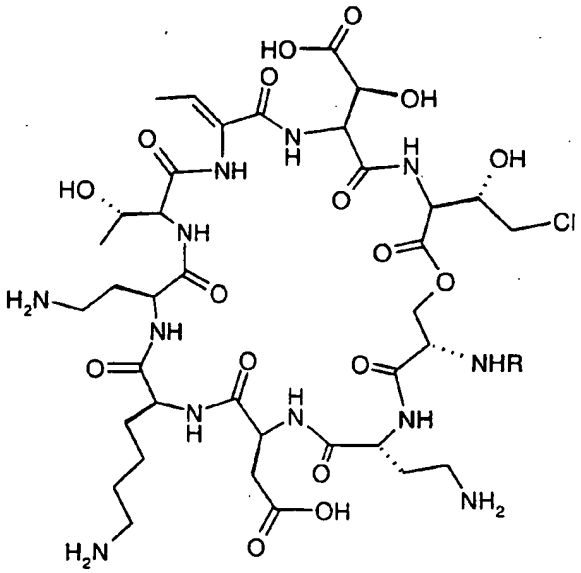
SUMMARY OF THE INVENTION

The present invention relates to a method and composition effective for administration of a pseudomycin or a related antifungal agent to a patient in need thereof. The method includes treating a fungal infection by administering an effective amount of a composition including a pseudomycin, such as pseudomycin B, or a related antifungal agent and hydroxypropyl-β-cyclodextrin or sulfobutylether-β-cyclodextrin. The method is effective to reduce symptoms of the fungal infection and, preferably, kills the fungus causing the infection. The method is effective against infections of fungi including *Candida spp.*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, and *Histoplasma capsulatum*. In a second embodiment, the method reduces adverse effects of injecting a pseudomycin, such as pseudomycin B, or a related antifungal agent by administering the pseudomycin or a related antifungal agent in a composition including hydroxypropyl-β-cyclodextrin or sulfobutylether-β-cyclodextrin. In another embodiment, the method includes administering toxicologically relevant doses of a pseudomycin, such as pseudomycin B, or a related antifungal agent in a composition including the pseudomycin and hydroxypropyl-β-cyclodextrin or sulfobutylether-β-cyclodextrin.

The composition of the method includes a pseudomycin or related antifungal agent, such as pseudomycin B, or a related antifungal agent and hydroxypropyl-β-cyclodextrin or sulfobutylether-β-cyclodextrin. Such a composition is effective for treating or reducing symptoms of fungal infections, for reducing adverse effects of injecting a pseudomycin, such as pseudomycin B, or a related antifungal agent and for administering toxicologically relevant doses of a pseudomycin, such as pseudomycin B, or a related antifungal agent. The composition includes the cyclodextrin in a molar excess over the pseudomycin or a related antifungal agent, preferably in at least about a two-fold molar excess and is suitable for parenteral administration. The composition can include excipients such as sodium chloride, mannitol, or dextrose which adjust the toxicity of the composition. The compositions containing a pseudomycin or a lipodepsidecapeptide antifungal agent, or a pharmaceutically acceptable salt, hydrate or ester thereof, in combination with a hydroxyalkyl-β-cyclodextrin or a sulfoalkylether-β-cyclodextrin may be used for the manufacture of a medicament for the treatment of a fungal infection.

Definitions

As used herein, the term "pseudomycin" refers to compounds having formula I:

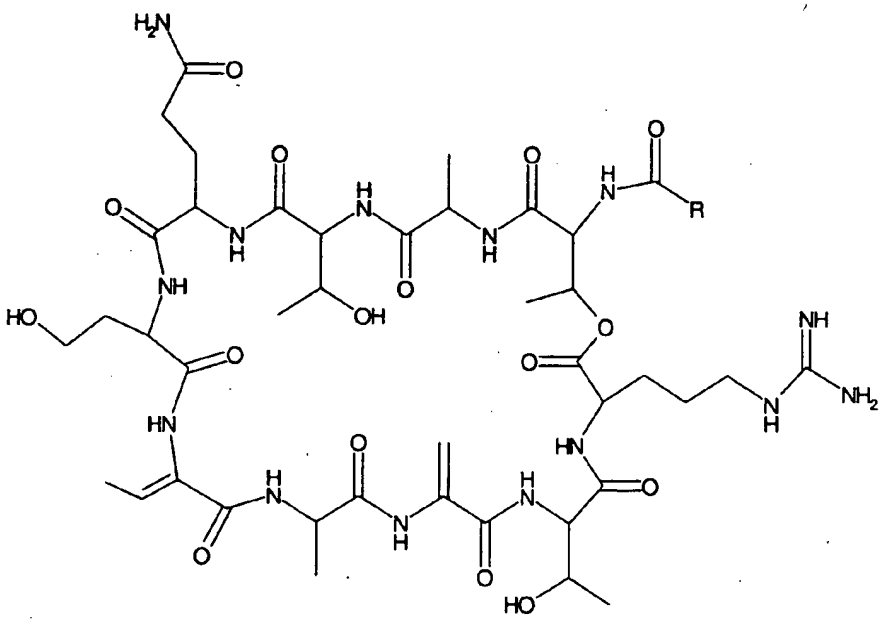


I

where R is a lipophilic moiety. The lipophilic moiety includes C_9-C_{15} alkyl, C_9-C_{15} hydroxyalkyl, C_9-C_{15} dihydroxyalkyl, C_9-C_{15} alkenyl, C_9-C_{15} hydroxyalkenyl, or C_9-C_{15} dihydroxyalkenyl. The pseudomycin compounds A, A', B, B', C, C' are represented by the formula I above where R is as defined below.

Pseudomycin A	R = 3,4-dihydroxytetradecanoyl
Pseudomycin A'	R = 3,4-dihydroxypentadecanoate
Pseudomycin B	R = 3-hydroxytetradecanoyl
Pseudomycin B'	R = 3-hydroxydodecanoate
Pseudomycin C	R = 3,4-dihydroxyhexadecanoyl
Pseudomycin C'	R = 3-hydroxyhexadecanoyl

As used herein, the term "lipodepsidecapeptide" refers to compounds having formula II:



II

where R is a lipophilic moiety. The lipophilic moiety includes C_9-C_{15} alkyl, C_9-C_{15} hydroxyalkyl, C_9-C_{15} dihydroxyalkyl, C_9-C_{15} alkenyl, C_9-C_{15} hydroxyalkenyl, or C_9-C_{15} dihydroxyalkenyl. Preferably, the lipophilic moiety is C_{11} alkyl. The alkyl, hydroxyalkyl, dihydroxyalkyl, alkenyl, hydroxyalkenyl, or dihydroxyalkenyl groups may be branched or

unbranched. Preferably, the amino acid sequence of the depsidecapeptide ring is threonine-alanine-threonine-glutamine-homoserine-dehydroaminobutyric acid-alanine-dehydroalanine-threonine-arginine, referred to herein as "25-B1 decapeptide" or "Thr-Ala-Thr-Gln-Xaa-Xaa-Ala-Xaa-Thr-Arg (SEQ ID NO: 1)".

As used herein, the term "25-B1 decapeptide antifungal agent A" refers to the specific depsidecapeptide having the preferred amino acid sequence SEQ ID NO: 1 and R is an unbranched C_{11} alkyl (i.e., R = $-(CH_2)_{10}CH_3$).

Any derivatives or analogs of the above described pseudomycins and related-pseudomycins (e.g., lipodepsidecapeptides) are also considered to be within the spirit of the present invention. Although a specific conformation is depicted above, other stereoisomers and diastereoisomers are also contemplated to be within the spirit of the present invention.

DETAILED DESCRIPTION

Pseudomycins and Related Antifungal Agents

As used herein, pseudomycin (PS) refers to one or more members of a family of antifungal agents that has been isolated from the bacterium *Pseudomonas syringae*. A pseudomycin is a lipodepsipeptide, a cyclic peptide including one or more unusual amino acids and having one or more appended hydrophobic or fatty acid side chains. Specifically, the pseudomycins are lipodepsinanopeptides, with a cyclic peptide portion closed by a lactone bond and including the unusual amino acids 4-chlorothreonine (ClThr), 3-hydroxyaspartic acid (HOAsp), 2,3-dehydro-2-aminobutyric acid (Dhb), and 2,4-diaminobutyric acid (Dab). It is believed that these unusual amino acids are involved in biological characteristics of the pseudomycins, such as stability in serum and their killing action. Pseudomycins include pseudomycin A, pseudomycin A', pseudomycin B (PSB), pseudomycin B', pseudomycin C, and pseudomycin C'. Each of these pseudomycins has the same cyclic peptide nucleus, but they differ in the hydrophobic side chain attached to this nucleus.

Pseudomycins A, A', B, B', C and C' have each been isolated and purified and their structures have been characterized by methods including sequencing, NMR, and

mass spectrometry. See, e.g., U.S. Patent No. 5,576,298, issued November 19, 1996 to Strobel et al. Harrison et al.); "Pseudomycins, a family of novel peptides from Pseudomonas Syringae possessing broad-spectrum antifungal activity" *J. Gen. Microbiology* **137**, 2857-2865 (1991); Ballio et al., "Novel bioactive lipodepsipeptides from *Pseudomonas syringae*: the pseudomycins" *FEBS Lett.* **355**, 96-100 (1994); and Coiro, V.M., et al., "Solution conformation of the *Pseudomonas syringae* MSU 16H phytotoxic lipodepsipeptide Pseudomycin A determined by computer simulations using distance geometry and molecular dynamics from NMR data," Eur. J. Biochem., **257**(2), 449-456 (1998). Antifungal activity due to pseudomycins was traced to *P. syringae* bearing a transposon known as Tn 903, which encodes kanamycin resistance. The sequence of and methods for manipulating transposon Tn 903 are known. *Oka et al.* J. Mol. Biol. 147, 217-226 (1981); "Nucleotide sequence of the kanamycin resistance transposon Tn 903." Methods for growth of various strains of *P. syringae* and their use in production of antifungal agents such as pseudomycins are disclosed in U.S. Patent Application Serial No. PCT/US00/08728 by Matthew D. Hilton, et al. entitled "Pseudomycin Production By *Pseudomonas Syringae*" submitted evendate herewith and described below for the production of the pseudomycins and lipodepsidecapeptides from biological materials on deposit (see Examples). Pseudomycins A' and B' are described in U.S. Patent Application Serial No. PCT/US00/08727, by Palaniappan Kulanthaivel et al. entitled " Pseudomycin Natural Products" submitted evendate herewith and may be produced using the general procedures described below. Each of the references cited in this paragraph is specifically incorporated herein by reference.

The pseudomycins vary in structure and properties. The pseudomycins exhibit activity against a wide variety of fungi and also exhibit generally acceptable toxicity. Compared to the other pseudomycins, pseudomycin B has greater potency against certain fungi and a lower level of toxicity. Therefore, for the present formulations and methods, pseudomycin B is preferred. The structure of pseudomycin B is represented by Formula I above where R =

3-hydroxytetradecanoyl. Pseudomycin B has a cyclic nonapeptide ring having the sequence Ser-Dab-Asp-Lys-Dab-Thr-Dhb-HOAsp-ClThr, more specifically, L-Ser-D-

Dab-L-Asp-L-Lys-L-Dab-L-aThr-Z-Dhb-L-Asp(3-OH)-L-Thr(4-Cl) with the carboxyl group of the ClThr and the hydroxyl group of the serine closing the ring with a lactone bond. The amine group of the serine forms an amide bond with the carboxyl of a 3-monohydroxytetradecanoyl moiety. The carboxyl group of the serine forms an amide bond with the Dab of the ring.

As used herein, antifungal agents related to pseudomycins include lipodepsidecapeptide antifungal agents produced by *Pseudomonas syringae*. A representative of this class of compounds, 25-B1 decapeptide antifungal agent A, has been purified and its structure determined. Lipodepsidecapeptide antifungal agents, such as 25-B1 decapeptide antifungal agent A, are described in U.S. Patent Application Serial No. PCT/US00/08724, by Palaniappan Kulanthaivel et al. entitled "Antifungal Agents Isolated From *Pseudomonas Syringae*" submitted evendate herewith and may be produced using the general procedures described below. This patent application is specifically incorporated herein by reference.

Method for Pseudomycin Production

To produce one or more pseudomycins from a wild type or mutant strain of *P. syringae*, the organism is cultured with agitation in an aqueous nutrient medium including an effective amount of three or fewer amino acids. The three or fewer amino acids are preferably glutamic acid, glycine, histidine, or a combination thereof. In one preferred embodiment, the amino acids include glycine and, optionally, one or more of a potato product and a lipid. Culturing is conducted under conditions effective for growth of *P. syringae* and production of the desired pseudomycin or pseudomycins. Effective conditions include temperature of about 22°C to about 27°C, and a duration of about 36 hours to about 96 hours. When cultivated on the media such as those described herein, *P. syringae* can grow in cell densities up to about 10-15 g/L dry weight and produce pseudomycins in total amounts at least about 10 μg/mL, preferably at least about 40 μg/mL, more preferably about 500 μg/mL or more.

Controlling the concentration of oxygen in the medium during culturing of *P. syringae* is advantageous for production of a pseudomycin. Preferably, oxygen levels are

maintained at about 5% to about 50% saturation, more preferably about 30% saturation. Sparging with air, with pure oxygen, or with gas mixtures including oxygen can regulate the concentration of oxygen in the medium. Further, adjustment of the agitation rate can be used to adjust the oxygen transfer rate.

Controlling the pH of the medium during culturing of *P. syringae* is advantageous for production of a pseudomycin. Pseudomycins are labile at basic pH, and significant degradation can occur if the pH of the culture medium is above about 6 for more than about 12 hours. Preferably, the pH of the culture medium is maintained at less than about 6, more preferably less than about 5.5, and most preferably above 4.0. The pH is preferably maintained at about 5 to about 5.4, more preferably about 5.0 to about 5.2. Although not limiting to the present invention, it is believed that pseudomycin degradation at basic pH is due to opening of the lactone ring and leaving of Cl^-.

P. syringae can produce one or more pseudomycins when grown in batch culture. However, fed-batch or semi-continuous feed of glucose and, optionally, an acid or base, such as ammonium hydroxide, to control pH, enhances pseudomycin production. Pseudomycin production by *P. syringae* can be further enhanced by using continuous feed methods in which glucose and, optionally, an acid or base, such as ammonium hydroxide, to control pH, are fed automatically.

Employing *P. syringae*, one or more pseudomycins can be produced in substantial quantities. That is, total quantities of one or more pseudomycins from at least about 200 μg/mL to about 900 μg/mL, preferably of from about 600 μg/mL to about 900 μg/mL, more preferably from about 800 μg/mL to about 900 μg/mL. Preferably, for production of pseudomycin A, pseudomycin A is produced in total quantities from at least about 10 μg/mL to about 400 μg/mL, more preferably of from about 300 μg/mL to about 400 μg/mL, most preferably from about 350 μg/mL to about 400 μg/mL. Preferably, for production of pseudomycin B, pseudomycin B is produced in total quantities from at least about 10 μg/mL to about 300 μg/mL, more preferably from about 200 μg/mL to about 300 μg/mL, most preferably from about 250 μg/mL to about 300 μg/mL. Preferably, for production of pseudomycin C, pseudomycin C is produced in total quantities from at least about 5 μg/mL to about 100 μg/mL, more preferably of from about 5 μg/mL to about 50

μg/mL, most preferably from about 10 μg/mL to about 50 μg/mL. Preferably, for production of pseudomycin C', pseudomycin C' is produced in total quantities from at least about 1 μg/mL to about 50 μg/mL, more preferably of from about 10 μg/mL to about 50 μg/mL, most preferably from about 30 μg/mL to about 50 μg/mL. Effective conditions for the production of Pseudomycin A' and/or B' include temperature of about 22°C to about 27°C, and a duration of about 36 hours to about 96 hours. When cultivated on the media such as those described herein, *P. syringae* can grow in cell densities up to about 10-15 g/L dry weight and produce pseudomycins A' and/or B' in total amounts at least about 10 μg/mL.

Choice of *P. syringae* strain can affect the amount and distribution of pseudomycin or pseudomycins produced by culturing under the conditions described herein. For example, strains MSU 16 H and 67 H1, and like strains, each produce predominantly pseudomycin A, but also produce pseudomycin B and C, typically in ratios of roughly 4:2:1. Strain 67 H1 and like strains, however, typically produce levels of pseudomycins about 3- to about 5-fold larger than are produced by strain MSU 16H. Compared to strains MSU 16 H and 67 H1, strain 25-B1 and like strains produce more pseudomycin B and less pseudomycin C. Strain 7H9-1 and like strains are distinctive in producing predominantly pseudomycin B and for producing larger amounts of pseudomycin B than other strains. For example this strain can produce pseudomycin B in at least a 10-fold excess over either pseudomycin A or C. Pseudomycin A' and/or B' can be produced from mutants of *P. syringae*. The mutants are produced by treating the bacteria with an amount of a mutagenic agent effective to produce mutants that overproduce pseudomycin A' and/or B', that produce pseudomycin A' and/or B' in excess over other pseudomycins, or that produce pseudomycin A' and/or B' under advantageous growth conditions. While the type and amount of mutagenic agent to be used can vary, a preferred method is to serially dilute NTG to levels ranging from 1 to 100 μg/ml. The mutants overproduce pseudomycin A' and/or B' preferably to at least about 10 μg/mL.

Each pseudomycin or mixtures of pseudomycins can be detected, determined, isolated, and/or purified by any of a variety of methods known to those of skill in the art. For example, the level of pseudomycin activity in a broth or in an isolated or purified

composition can be determined by antifungal action against a fungus such as Candida. Numerous methods are known for the preparation and analysis of the pseudomycins. For example, one or more pseudomycins can be isolated and purified by chromatography, such as HPLC.

Method for Producing a *P. syringae* Lipodepsidecapeptide

To produce one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, from a wild type or mutant strain of *P. syringae*, the organism is cultured with agitation in an aqueous nutrient medium including an effective amount of three or fewer amino acids. The three or fewer amino acids are preferably glutamic acid, glycine, histidine, or a combination thereof. In one preferred embodiment, the amino acids include glycine and, optionally, one or more of a potato product and a lipid. Culturing is conducted under conditions effective for growth of *P. syringae* and production of a desired *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A. Effective conditions include a temperature of about 22° C to about 27° C, and a duration of about 36 hours to about 96 hours. When cultivated on media such as those described herein, *P. syringae* can grow at cell densities up to about 10-15 g/L dry weight and produce a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, in a total amount at least about 10 μg/mL, preferably at least about 50 μg/mL.

Controlling the concentration of oxygen in the medium during culturing of *P. syringae* is advantageous for production of a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A. Preferably, oxygen levels are maintained at about 5% to about 50% saturation, more preferably about 30% saturation. Sparging with air, with pure oxygen, or with gas mixtures including oxygen can regulate the concentration of oxygen in the medium. Further, adjustment of the agitation rate can be used to adjust the oxygen transfer rate.

Controlling the pH of the medium during culturing of *P. syringae* is advantageous for production of a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide

antifungal agent A. The pH of the culture medium can be maintained at less than about 6 and above about 4.

P. syringae can produce a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, when grown in batch culture. However, fed-batch or semi-continuous feed of glucose and, optionally, an acid or base, such as ammonium hydroxide, to control pH, enhances production of a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A. Production of a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, by *P. syringae* can be further enhanced by using continuous culture methods in which glucose and, optionally, an acid or base, such as ammonium hydroxide, to control pH, are fed automatically. The pH is preferably maintained at a pH of about 5 to about 5.4, more preferably about 5.0 to about 5.2.

Choice of *P. syringae* strain can affect the amount and distribution of a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, produced by culturing under the conditions described herein. For example, strain 25 B1 can produce predominantly 25-B1 decapeptide antifungal agent A.

The cyclic decapeptide nucleus of the *P. syringae* lipodepsidecapeptides can be prepared by cleaving off the lipophilic moiety, such as by deacylation. Cleavage and deacylation methods are well-known to those skilled in the art, such as the use of deacylase enzymes.

Cyclodextrins

As used herein, "cyclodextrin" refers to a compound including cyclic alpha(1→4) linked D-glucopyranose units. Typically, cyclodextrins are formed by the action of an amylase on starch. α-cyclodextrin refers to a cyclodextrin with 6 cyclic, linked D-glucopyranose units, β-cyclodextrin has 7 cyclic, linked D-glucopyranose units, and γ-cyclodextrin has 8 cyclic, linked D-glucopyranose units. These cyclic, linked D-glucopyranose units define a hydrophobic cavity, and cyclodextrins are known to form inclusion compounds with other organic molecules, with salts, and with halogens either in the solid state or in aqueous solutions. Most natural, unsubstituted cyclodextrins exhibit

unacceptable toxicity in pharmaceutical compositions and are not readily eliminated once in a patient's blood stream. Methods for preparing cyclodextrins are well-known to those skilled in the art.

Cyclodextrins include substituted cyclodextrins. As used herein, "substituted cyclodextrin" refers to a derivative of a cyclodextrin formed by addition of a side chain to one or more positions on the cyclodextrin. The side chain can be an organic moiety, or a heteroorganic moiety. Substituted cyclodextrins include cyclodextrins that have been alkylated, hydroxy alkylated or reacted to form a sulfoalkyl ether. Substituted cyclodextrins include hydroxypropyl cyclodextrin, hydroxyethyl cyclodextrin, glucosyl cyclodextrin, maltosyl cyclodextrin, and sulfobutylether cyclodextrin. Methods for preparing cyclodextrin derivatives are known in the art, and certain methods are described in U.S. Patent Nos. 4,727,064 (issued to Pitha on February 23, 1988) and 5,134,127 (issued to Stella et al. on July 28, 1992), European Patent Application Publication Number 0 499 322 A1, and Pitha et al., Int. J. Pharm., **29**, 73-82 (1986). The disclosures of each of the documents cited in this paragraph is specifically incorporated herein by reference.

The cyclodextrins vary in structure and properties. For example, the size (e.g. diameter, and depth) and functionality (e.g. hydrophobicity, charge, reactivity, and ability to hydrogen bond) of the hydrophobic cavity varies among the substituted and unsubstituted α-, β-, and γ-cyclodextrins. Typically, a cyclodextrin selected for a formulation has a size and functionality that compliments the other components of the formulation. For the present formulations and methods, it is believed that substituted cyclodextrins like the hydroxyalkyl cyclodextrins and sulfoalkylether cyclodextrins have a size and functionality that compliment the other components of the formulation, such as the pseudomycin or related antifungal agent, such as pseudomycin B. Preferred cyclodextrins include hydroxypropyl-β-cyclodextrin (HP-CD), and sulfobutylether-β-cyclodextrin (SBE-CD).

Sulfobutylether cyclodextrin is commercially available and can be synthesized by methods known in the art. As used herein, sulfobutylether cyclodextrin or SBE-CD refers to the sulfobutylether derivative of β-cyclodextrin typically including an average of about

1 to about 8, preferably about 6 to about 7 sulfobutylether groups at the 2, 3, and 6 positions on the β-cyclodextrin ring. Hydroxypropyl cyclodextrin is commercially available and can be synthesized by methods known in the art. As used herein, hydroxypropyl cyclodextrin or HP-CD refers to the 2-hydroxypropyl derivative of β-cyclodextrin typically including an average of about 8 2-hydroxypropyl groups at 2, 3, and 6 positions on the β-cyclodextrin ring.

Biological Activities of Pseudomycins and Related Antifungal Agents

Pseudomycins have several biological activities including killing various fungi, such as fungal pathogens of plants and animals. In particular, pseudomycins or related antifungal agents are active antimycotic agents against fungi that cause opportunistic infections in immune compromised individuals. These fungi include *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Histoplasma capsulatum*, and various species of *Candida* including *C. parapsilosis*, *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. krusei*. Killing, rather than inhibiting the growth of fungi, particularly of fungal pathogens, is a desirable and preferred biological activity of a pseudomycin.

The pseudomycins have also been shown to be toxic to a broad range of plant-pathogenic fungi including *Rynchosporium secalis, Ceratocystis ulmi, Rizoctonia solani, Sclerotinia sclerotiorum, Verticillium albo-atrum, Verticillium dahliae, Thielaviopis basicola, Fusarium oxysporum and Fusarium culmorum*. (see Harrison, L., et al., "Pseudomycins, a family of novel peptides from *Pseudomonas syringae* possessing broad-spectrum antifungal activity," *J of General Microbiology*, 7, 2857-2865 (1991).) In addition, *P. syringae* MSU 16H has been shown to confer a greater protection than the wild-type strain in elms infected with *Ceratocystic ulmi*, the causal agent of Dutch elm disease. (see e.g., Lam et al, *Proc. Natl. Sci. USA*, 84, 6447-6451 (1987)).

Pseudomycins or related antifungal agents have certain adverse biological activities, or adverse effects. As used herein, adverse effects of a pseudomycin or related antifungal agent refers to adverse effects occurring at or near the site of injection of a pseudomycin or related antifungal agent. Of particular interest are the adverse effects of a pseudomycin, such as pseudomycin B, or a related antifungal agent. For example,

intravenous injection of a pseudomycin, such as pseudomycin B, in an acetate buffer formulation results in damaging the vein into which the pseudomycin was injected. Outside the vein (i.e., extravasation), pseudomycin damages tissue near the site of injection. The adverse effects of a pseudomycin, such as pseudomycin B, on the vein and on surrounding tissues include destruction of the endothelium of the vein, destruction of tissue, inflammation, and local toxicity to host tissues. Although not limiting to the present invention, it is believed that intravenous injection of the pseudomycin results in loss of vascular endothelium and extravasation of the pseudomycin which, in turn, results in adverse effects on the tissues around the vein.

Formulation and Antifungal Action of a Pseudomycin or Related Antifungal Agent

Adverse effects result from injection of numerous different formulations of a pseudomycin, such as pseudomycin B, or a related antifungal agent including an acetate buffer formulation, a saline formulation, a microemulsion formulation, an emulsion formulation, a Povidone K12 complex formulation, and a micelle formulation. The observed adverse effects with these vehicles prevent administration of effective antifungal amounts of the pseudomycin. The adverse effects typically require halting dosing with the pseudomycin or related antifungal agent after only one or a few injections, and before a sufficient antifungal effect is obtained. Even in their milder forms, such adverse effects at the site of injection can discourage patient compliance with an effective dosing regime. Therefore, an effective antifungal method employing pseudomycin or related antifungal agent requires a formulation that allows administration of repeated doses. Such a formulation permits administration of repeated doses of pseudomycin or related antifungal agent of at least about 0.01 mg/kg, preferably more than about 0.1 mg/kg, preferably about 0.1 to about 5 mg/kg, more preferably about 0.1 to about 0.5 mg/kg, without unacceptable adverse effects at the site of injection.

The observed adverse effects also prevent administration of doses of a pseudomycin, such as pseudomycin B, or a related antifungal agent approaching the maximum tolerated dose, that is, the lowest dose that causes significant systemic toxicity or target organ toxicity in the host. Such high doses of pseudomycin are typically about

0.5 mg/kg, preferably about 5 mg/kg or more, preferably about 5 to about 25-50 mg/kg. Even a single administration of pseudomycin in vehicles such as an acetate buffer can cause adverse effects that prevent further administration of pseudomycin or related antifungal agent. Such adverse effects can prevent administration of a toxicologically relevant dose of pseudomycin or related antifungal agent, which prevents determining relevant toxicological parameters for the antifungal agent. Such high doses also become necessary for therapy of fungal infections for strains or organisms tolerant of or resistant to a pseudomycin, such as pseudomycin B, or a related antifungal agent.

Formulating a pseudomycin, such as pseudomycin B, or related antifungal agent with a cyclodextrin, such as HP-CD, SBE-CD, or a like cyclodextrin, allows effective antifungal or toxicological dosing of the agent. Such a formulation reduces or prevents adverse effects of injection of the pseudomycin or related antifungal agent. Pseudomycin B is soluble in aqueous solution to at least about 15 mg/mL, so the cyclodextrin is not typically needed to increase the solubility of this antifungal agent. Pseudomycin B and HP-CD or SPE-CD can be dissolved without difficulty at suitable concentrations in an aqueous vehicle such as saline for injection, or known mannitol or dextrose vehicles. The toxicity of the formulation can be adjusted with, for example, NaCl to bring it within an appropriate physiological range, if necessary.

Preferably, a formulation of a pseudomycin, such as pseudomycin B, or a related antifungal agent with HP-CD, SBE-CD, or a like cyclodextrin includes about 0.05 mg/mL to about 20 mg/mL of pseudomycin, preferably about 1 to about 15 mg/mL. The formulation includes HP-CD, SBE-CD, or a like cyclodextrin in an amount effective to reduce the adverse effects of injection of pseudomycin or related antifungal agent and to allow toxicological or antifungal dosing of this antifungal agent. Typically, HP-CD, SBE-CD, or a like cyclodextrin are present in the formulation at about 0.5 wt-% (wt/vol of solution) to about 6 wt-%, preferably about 1 wt-% to about 5 wt-%, preferably about 2 wt-%. Advantageously, HP-CD, SBE-CD, or a like cyclodextrin is present in a molar excess over the pseudomycin, such as pseudomycin B, or a related antifungal agent, preferably, at least about a 2-fold to about a 4-fold molar excess. Dosing of such an effective toxicological or antifungal formulation can occur for at least about 1-5,

preferably about 2-3 injections per day for about 1-7 days without unacceptable adverse effects at the site of injection.

Although not limiting to the present invention, it is believed that the adverse effects of pseudomycins or related antifungal agents upon injection are due in part to effects of soluble aggregates of the agent. For example, a pseudomycin, such as pseudomycin B, or a related antifungal agent includes a peptide ring and a long fatty chain and it is believed a long fatty chain may induce the formation of micelles in aqueous solutions or in blood. According to this model, it is desirable to reduce the size of the soluble aggregates of the pseudomycin or related antifungal agent. This can be accomplished by manipulating the composition of the vehicle to reduce the impetus of the pseudomycin or related antifungal agent to aggregate. Alternatively, the vehicle can include an agent that complexes the pseudomycin or related antifungal agent and prevents the aggregates from damaging the vein and surrounding tissues. For example, a complex of the fatty chain of the pseudomycin or related antifungal agent with a cyclodextrin or substituted cyclodextrin, can, according to this model, provide a form of the pseudomycin or related antifungal agent that does not cause the adverse reaction at the site of injection.

When administered in an effective antifungal amount, a composition of a pseudomycin, such as pseudomycin B, or a related antifungal agent with a cyclodextrin, such as HP-CD or SPE-CD, reduces the burden of a fungal infection, reduces symptoms associated with the fungal infection, and can result in elimination of the fungal infection. The fungal infection can be eliminated because pseudomycins can kill, rather than just reducing the growth of fungi.

A typical patient in need of antifungal therapy with a formulation of a pseudomycin, such as pseudomycin B, or a related antifungal agent and HP-CD, SBE-CD, or a like cyclodextrin has severe symptoms of infection, such as high fever, and is likely to be in intensive or critical care. Various fungi can cause such serious infections. *Candida*, for example, causes mucosal and serious systemic infections and exists in strains that are resistant to azole and polyene antifungals. *Aspergillus* causes life-threatening systemic infections. *Cryptococcus* is responsible for meningitis. Such serious fungal infections may occur in immune compromised patients, such as those receiving

organ or bone marrow transplants, undergoing chemotherapy for cancer, recovering from major surgery, or suffering from HIV infection.

The antifungal therapy includes administration, typically parenteral administration, preferably intravenous administration, of a formulation of a pseudomycin, such as pseudomycin B, or a related antifungal agent and HP-CD, SBE-CD, or a like cyclodextrin over several days to halt the infection. For most fungal infections reduction of symptoms of the infection includes reduction of fever, return to consciousness, and increased well being (e.g. the patient acts and feels better) of the patient. Preferably, symptoms are reduced by killing the fungus to eliminate the infection or to bring the infection to level tolerated by the patient or controlled by the patient's immune system.

Pharmaceutical Compositions Including a Formulation of a Pseudomycin or Related Antifungal Agent and a Cyclodextrin

A pharmaceutical composition including a formulation of a pseudomycin, such as pseudomycin B, or a related antifungal agent and a cyclodextrin, such as SBE-CD or HP-CD, can also include carriers, excipients, vehicles, and other additives. The formulation can include additives such as various oils, including those of petroleum, animal, vegetable or synthetic origin, for example, peanut oil, soybean oil, mineral oil, and sesame oil. Suitable pharmaceutical excipients include starch, cellulose, glucose, lactose, sucrose, gelatin, malt, magnesium stearate, sodium stearate, glycerol monostearate, sodium chloride, dried skim milk, glycerol, propylene glycol, water, and ethanol. The compositions can be subjected to conventional pharmaceutical expedients, such as sterilization, and can contain conventional pharmaceutical additives, such as preservatives, stabilizing agents, wetting, or emulsifying agents, salts for adjusting osmotic pressure, and buffers. Suitable pharmaceutical carriers and their formulations are described in Martin, "Remington's Pharmaceutical Sciences," 15th Ed.; Mack Publishing Co., Easton (1975); see, e.g., pp. 1405-1412 and pp. 1461-1487. Such compositions will, in general, contain an effective amount of the active compound together with a suitable amount of carrier so as to prepare the proper dosage form for proper administration to the host.

Typically, the compositions will be administered to a patient (human or other animal, including mammals such as, but not limited to, cats, horses and cattle and avian species) in need thereof, in an effective amount to inhibit the fungal replication. For systemic administration, the daily dosage as employed for adult human treatment will range from 5 mg to 5000 mg of active ingredient, preferably 50 mg to 2000 mg, which can be administered in 1 to 5 daily doses, for example, depending on the route of administration and the condition of the patient. When the compositions include dosage units, each unit will preferably contain 2 mg to 2000 mg of active ingredient, for example 50 mg to 500 mg. For serious infections, the compound can be administered by intravenous infusion using, for example, 0.01 to 10 mg/kg/hr of the active ingredient.

Uses of Pseudomycin-Cyclodextrin Formulations

The present invention also encompasses a kit including the present pharmaceutical compositions and to be used with the methods of the present invention. The kit can contain a vial which contains a formulation of the present invention and suitable carriers, either dried or in liquid form. The kit further includes instructions in the form of a label on the vial and/or in the form of an insert included in a box in which the vial is packaged, for the use and administration of the compounds. The instructions can also be printed on the box in which the vial is packaged. The instructions contain information such as sufficient dosage and administration information so as to allow a worker in the field to administer the drug. It is anticipated that a worker in the field encompasses any doctor, nurse, or technician who might administer the drug.

The present invention also relates to a pharmaceutical composition including a formulation of a pseudomycin, such as pseudomycin B, or a related antifungal agent and a cyclodextrin, and that is suitable for administration by injection. According to the invention, a formulation of a pseudomycin, such as pseudomycin B, or a related antifungal agent and a cyclodextrin can be used for manufacturing a composition a pseudomycin, such as pseudomycin B, or a related antifungal agent and a cyclodextrin in a form that is suitable for administration by injection. For example, a liquid or solid formulation can be manufactured in several ways, using conventional techniques. A

liquid formulation can be manufactured by dissolving a pseudomycin, such as pseudomycin B, or a related antifungal agent and cyclodextrin in a suitable solvent, such as water, at an appropriate pH, including buffers or other excipients.

Agricultural Compositions Including a Formulation of a Pseudomycin or Related Antifungal Agent and a Cyclodextrin

Compositions containing one or more pseudomycin, lipodepsidecapeptide, or combinations thereof (including hydrates, solvates, and esters thereof) and a cyclodextrin (e.g., SBE-CD or HP-CD) may be used in the treatment of fungi in plants (in particular, *V. albo-atrum, Rhizoctonia solani and F. oxysporum*) either as a direct treatment or preventative treatment. Generally, the infected plants are treated by injecting or spraying an aqueous suspension of the *P. syringae* antifungal agents into or onto the plant. Means of injection are well-known to those skilled in the art (e.g., gouge pistol). Any means of spraying the suspension may be used that distributes an effective amount of the active material onto the plant surface. The suspension may also include other additives generally used by those skilled in the art, such as solubilizers, stabilizers, wetting agents, and combinations thereof.

The present invention may be better understood with reference to the following examples. These examples are intended to be representative of specific embodiments of the invention, and are not intended as limiting the scope of the invention.

EXAMPLES

Biological Materials on Deposit

P. syringae MSU 16H is publicly available from the American Type Culture Collection, Parklawn Drive, Rockville, MD, USA as Accession No. ATCC 67028. *P. syringae* strains 25-B1, 7H9-1, and 67 H1 were deposited with the American Type Culture Collection on March 23, 2000 and were assigned the following Accession Nos.:

25-B1	Accession No. PTA-1622
7H9-1	Accession No. PTA-1623
67 H1	Accession No. PTA-1621

Example 1 -- Adverse Reaction to Intravenous Injection of a Formulation of Pseudomycin B in Saline for Injection and Without Cyclodextrin

Pseudomycin B formulated in saline for injection, without any cyclodextrin, was evaluated in a 1-week pilot study for adverse reaction upon intravenous injection.

Materials and Methods

CD-1 mice (males) were divided into groups of four and were given 0, 1, 5, 10, 25, or 50 mg /kg/day PSB-TFA as an intravenous injection (slow bolus) into a lateral tail vein. The vehicle used for this study was saline for injection. The potency of the trifluoroacetic acid salt of pseudomycin B (PSB-TFA) was adjusted to account for the trifluoroacetic acid present in the salt form. The data collected included live phase (i.e., clinical observations, body weight, and mortality), clinical chemistry, limited organ weights, and limited histopathology (kidney, liver, heart, and injection site).

Results

All animals survived to the scheduled termination on day 7. However, each of the mice exhibited a severe adverse reaction at the site of injection. This began as marked swelling and darkening of the tail, typically occurring within about 6 to 8 hours after injection. The severity of the tissue reaction in all animals receiving a dose of $\geq$ 10 mg/kg resulted in discontinuation of dosing after 2 to 5 doses. There were no significant clinical chemistry, gross pathology, or histopathology findings observed to indicate target organ effects other than evidence of severe adverse reaction at the injection site.

Example 2 --Evaluation of the Adverse Reaction of Animal Cells to Pseudomycin B and Related Compounds

Pseudomycin B and related compounds were tested for their *in vitro* toxicity toward mammalian cells to evaluate whether toxicity was related to the structure of the compound.

Materials and Methods

Pseudomycin B (PSB) and the related compound pseudomycin A (PSA) were tested at a concentration of 0.5 mg/mL for their effect on L6 skeletal muscle cells. This in vitro muscle model has been demonstrated to correlate with in vivo parenteral antibiotic venous irritation. See: Rosalki S.B., An Improved Procedure for Serum Creatine Phosphokinase Determination. J. Lab. Clin. Chem. **69**: 696-705 (1967); D.M. Hoover et al., Fundamental and Applied Toxic. **14**: 589-597 (1990); Mosmann T., Rapid Colorimetric Assay for Cellular Growth and Survival, J. Immunol. Method **65**: 55-63 (1983).

Toxicity was determined by monitoring the levels of creatine phosphokinase (CPK) and MTT reductase activities in the cells by standard methods.

Results

The data are summarized in Table 1 which identifies the compounds tested and reports the amount of creatine phosphokinase retention (percent of control), and the MTT reductase activity (percent of control). The greater the toxicity the lower the measured levels of CPK retention and MTT reductase activity.

Table 1 – *In Vitro* Toxicity of Pseudomycin B and Related Compounds.

Compound Description	CPK	MTT
PSB-TFA	0	13
PSB-FB	0	10
PSC	0	3
PSA	0	11

Conclusion

This in vitro cellular toxicity of pseudomycins A and B, and suggests that formulation of these compounds may be necessary to reduce adverse reactions upon intravenous injection.

Example 3 -- Evaluation of Adverse Reactions and Their Amelioration Upon Intravenous Injection of Various Formulations of Intravenous Pseudomycin B

These three studies examined the effects of various formulations of pseudomycin B on the adverse reactions observed at the site of injection; and amelioration of the adverse reaction, upon intravenous injection.

Study 1

This study evaluated formulations including pseudomycin B in an acetate buffer, in a microemulsion, and with SBE-CD.

Materials and Methods

Animals were as described above in Example 1. The doses selected were based on the adverse effects observed in Figure 1. The low dose was 5 mg/kg, which, in Example 1, caused slight tissue injury but allowed dosing for 7 full days. The high dose was 25 mg/kg, which, in Example 1, resulted in severe tissue injury and restricted dosing. This study tested three formulations acetate buffer, a microemulsion vehicle, and a sulfobutylether-β-cyclodextrin (SBE-CD) vehicle at each of these two doses of PSB-TFA. The microemulsion vehicle includes 1.7 wt-% propylene glycol, 3.4 wt-% emulphor EL, 1.4 wt-% phospholipon 90, 3.5 wt-% fractionated coconut oil in 0.05M acetate buffer with 1.75 wt-% dextrose, at pH 4.5.

SBE-CD was obtained from Cydek, Inc., 12980 Metcalf Ave., Suite 470, Overland Park, Kansas 66213 and was prepared as 2 wt-% Sulfobutylether-β-cyclodextrin in 0.05M acetate buffer with 1.75 wt-% dextrose, pH 4.5. Typically a SBE-CD stock solution is prepared by mixing glacial acetic acid with deionized water and NaOH to achieve a solution that is 0.05 M in acetic acid and at pH 4.5. Solid dextrose and SBE-CD are dissolved in the acetic acid buffer to the desired concentration. Then pseudomycin B is added to achieve the desired concentration and the solution is stirred until the pseudomycin dissolves.

Results

The study design and results are summarized in the Table 2.

Table 2 -- Reactions to Various Formulations of Pseudomycin B (PSB).

Group	Vehicle	Dose of PSB	Results
01	acetate buffer	5 mg/kg	Received full 7 doses, tail swelling noted.
02	acetate buffer	25 mg/kg	Received 2-3 doses, tail swelling after first dose, bruising after second dose, and blackened tails by day 4. A portion of the tail from 2 animals was missing on day 5.
03	microemulsion[1]	5 mg/kg	Received full 7 doses, tails looked normal throughout study.
04	microemulsion[1]	25 mg/kg	Received 2 doses, tail swelling after first and second doses. The tails recovered to the point at which they could have been dosed again by day 5 but further recovery was monitored through the end of the study.
05	SBE cyclodextrin[2]	5 mg/kg	Received 7 full doses, tails looked normal throughout the study.
06	SBE cyclodextrin[2]	25 mg/kg	Received 7 full doses, tails looked normal throughout the study.

[1] 1.7 wt-% Propylene glycol, 3.4 wt-% Emulphor EL, 1.4 wt-% Phospholipon 90, 3.5 wt-% fractionated coconut oil in 0.05M acetate buffer with 1.75 wt-% dextrose, pH 4.5

[2] 2 wt-% Sulfobutylether-β-cyclodextrin in 0.05M acetate buffer with 1.75 wt-% dextrose, pH 4.5

Conclusion

The results of this study indicate that SBE-CD vehicle completely ameliorated the adverse effects exhibited by pseudomycin B upon intravenous administration of doses up to 25 mg/kg. The microemulsion reduced the adverse effects at both doses. However, at the higher doses, the adverse effects were sufficient that dosing had to be suspended.

Study 2

This study evaluated formulations including pseudomycin B in an acetate buffer, with SBE-CD, with HP-CD, with gamma-CD, in an emulsion, in a Povidone formulation, and in a micelle formulation.

Materials and Methods

Animals were as described in Example 1 and were used in groups of three. Pseudomycin B was administered at a dose of 25 mg/kg/day for 7 days by intravenous bolus administration into a lateral tail vein at 10 mL/kg. The vehicle for Group 1 (positive control) was acetate buffer alone since this has been shown (Example 1 and

Example 2, Study 1) to result in marked adverse reactions. The vehicle for Group 2 (negative control) was acetate buffer containing 2 wt-% SBE-CD which has been shown (Example 2, Study 1) to completely ameliorate the adverse effects observed when pseudomycin B is administered in acetate buffer. The other vehicles are as follows: Group 3, 2 wt-% hydroxypropyl-β-cyclodextrin; Group 4, 2 wt-% gamma-cyclodextrin; Group 5, lyposin II emulsion, which includes 10% soybean oil and 10% safflower oil; Group 6, Povidone K12 emulsion, which includes Polyvinylpyrrolidone 2000-3000 m.w.; and Group 7, 0.5 wt-% polysorbate 80 micelle preparation, which includes dextrose, polysorbate 80, pH 4.5 acetate buffer.

Results

The results of the study are shown in Table 3.

Table 3 -- Reactions to Various Formulations of Pseudomycin B.

Treatment	Day 1	Day 2	Day 3	Day 3	Day 4	Day 5	Day 6
0.05M acetate buffer [Positive Control]							
2 wt-% SBE- CD [Negative Control]							
2 wt-% Hydroxypropyl-CD							
2 wt-% Gamma-CD							
Lyposin II emulsion							
Povidone K12 complex							
0.5 wt-% Polysorbate 80 micelle preparation							

Normal dosing
Partial dosing and/or swelling/discoloration noted after dosing
No dosing

- Animals in acetate buffer, Povidone, and polysorbate groups exhibited severe tail vein irritation.
- Day 2 - One animal in the Gamma-CD group died due to a dosing accident. Another animal in this group exhibited reddening of its tail.
- Day 4 - The animal that had tail reddening on Day 2 was found dead due to causes unknown.

The results of this study confirmed the irritation potential of pseudomycin B when administered in acetate buffer. Group 1 showed swelling and discoloration of the tail after the first dose which impaired dosing on the second and third day. By day 4, the adverse reaction prevented further dosing of the animals. SBE-CD prevented the adverse reaction observed in Group 1. Group 2 received 7 daily doses of 25 mg/kg pseudomycin B with no evidence of adverse reactions. Groups 3 and 4, formulations including

hydroxypropyl-β-cyclodextrin or gamma-cyclodextrin, also showed protection from the adverse reactions caused by pseudomycin B. The Liposin II emulsion provided only partial protection. Although the animals were dosed daily for 7 days, tail swelling impaired dosing on the last 3 days of the study. The Povidone K12 complex and 0.5 wt-% Polysorbate 80 micelle preparation provided no protection compared with acetate buffer alone.

Conclusion

This study showed that additional cyclodextrins, such as hydroxypropyl-β-cyclodextrin and gamma-cyclodextrin also exhibit the protective effect. However, the results with gamma-CD may be equivocal due to the tail reddening and unexplained death seen with one animal in this group.

Study 3

This study evaluated formulations including pseudomycin B in saline for injection at two pHs, in pluronic F68, in solutol, and in polysorbate.

Materials and Methods

Animals were female CD1 mice handled according to established protocols. The mice were divided into groups of three. Pseudomycin B was administered at a dose of 25 mg/kg/day for 7 days by intravenous bolus administration into a lateral tail vein at 10 mL/kg. The vehicle for Group 2 was 0.9 wt-% NaCl (saline for injection) at pH 4.5. The vehicle for Group 3 was 0.9 wt-% NaCl (saline for injection) at pH 6.5. The vehicle for Group 4 was 0.9 wt-% NaCl in 0.05 wt-% Pluronic F68, which includes propylene oxide and ethylene oxide copolymers at various m.w. and HLB at pH 6.5. The vehicle for Group 5 was 0.9 wt-% NaCl in 0.5 wt-% Pluronic F68 at pH 6.5. The vehicle for Group 6 was 0.9 wt-% NaCl in 0.05 wt-% Solutol, which includes 70% polyglycol ester of 12-hydroxystearate and 30% polyethylene glycol at pH 6.5. The vehicle for Group 7 was 0.9 wt-% NaCl in 0.05 wt-% polysorbate 20 at pH 6.5.

Results

The results of the study are shown in Table 4. Clinical observations that affected all treatment groups included swollen and discolored tails. In addition, individual animals in Groups 03-07 demonstrated evidence of tissue necrosis. Three animals died on study (Animal 6051 on test day 5 and Animals 2053 and 6052 on test day 6). All of these deaths were the result of mechanical injuries sustained during dosing and, therefore, were not considered compound-related.

Table 4 -- Reactions to Various Formulations of Pseudomycin B.

Animal	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
2051	0	0	0	1	1	1	1,UD	1
2052	0	0	0	1	1	1	1	1
2053	0	0	0	1,2,UD	1	1	+	+
3051	1,2	1,2	3	1,2,3,PD	1,2,3,UD	1,2,3,UD	1,2,3,UD	1,2,3
3052	0	0	1	1,2,3,UD	1,2,3,UD	1,2,3,UD	1,2,3,UD	1,2,3
3053	0	1,2	1,2	1,2,3	1,2,3,UD	1,2,3,UD	1,2,3,UD	1,2,3
4051	0	1.2	3	1,2,3,UD	1,2,3,UD	1,2,3,UD	1,2,3,UD	1,2,3
4052	0	0	1	1,PD	1	1,2	1,2,PD	1,2
4053	0	0	1	1	1	1,2,UD	1,2,3	1,2,3
5051	0	0	1,2	1,2,3,UD	1,2,3,UD	1,2,3,UD	1,2,3,UD	1,2,3
5052	0	0	1,2	1,2,3	1,2,3,UD	1,2,3,UD	1,2,3,UD	1,2,3
5053	0	0	1	1	1	1,2	1,2,PD	1,2
6051	0	0	1	1	1	+	+	+
6052	0	0	1	1	1,PD	1,2,UD	+	+
6053	0	0	1	1,2,UD	1,2,3,UD	1,2,3,UD	1,2,3,UD	1,2,3
7051	0	1	1	1,3	1,2,3,UD	1,2,3,UD	1,2,3,UD	1,2,3
7052	0	1	1	1	1,UD	1,UD	1,UD	1,2
7053	0	1	3	1,3,UD	1,2,3,UD	1,2,3,UD	1,2,3,UD	1,2,3

0=Normal, 1=tail swollen, 2=tail discoloration, 3=tail necrotic, PD=partial dose, UD=unable to dose
+=Animals Dead
The gray shading highlights the point in the study at which tissue injury prevented even partial dose administration for at least one day.

Conclusion

None of the formulations tested in the current study completely ameliorated the adverse effects of injection of Pseudomycin B. Complete amelioration has been demonstrated with SBE-CD.

Example 4 -- *In vivo* Toxicological Evaluation of an SBE-CD Formulation with Varying Concentrations of Pseudomycin B

This study evaluated potential toxicities, including any adverse effects at the site of injection, in an animal model of formulations of sulfobutylether-β-cyclodextrin at four concentrations of pseudomycin B.

Materials and Method

The doses of pseudomycin B used for this rat study were 0, 1, 10, or 50 mg/kg/day for 14 days administered as an intravenous bolus dose. The SBE-CD formulation was 2 wt-% and the dose volume used was 5 mL/kg. Toxicological parameters monitored by standard methods included body weights, clinical observations, mortality, hematology, clinical chemistry, limited pathology (liver, kidney, heart, and injection site), and bone marrow micronucleus (as a preliminary assessment of genetic toxicity).

Results

Within 2 days of starting the live phase, the tails of the high-dose animals were swollen and discolored to the point that further dosing became impossible (Table 5). The decision was made to pull these animals off study and to reevaluate the method and formulation used for high-dose toxicity assessment. The 1 and 10 mg/kg/day dose groups went the full 2 weeks and showed no evidence of overt toxicity.

Table 5 -- Results of Administering SBE-CD Formulations at Three Concentrations of Pseudomycin B.

PARAMETERS	1 mg/kg/day	10 mg/kg/day	50 mg/kg/day
Clinical Observations	-	-	· **Group terminated on study day 3** due to excessive tail vein irritation
Mortality	-	-	-
Body Weight	-	-	· ↓BW
Gross Pathology	-	-	-
Organ Weights (Li, Ki, and Ht)	-	-	· ↑Ki weight · ↑Li weight
Hematology	-	-	
Clinical Chemistry	-	-	
Histopathology (Li, Ki, Ht, and IS)	-	-	· Slight vacuolization of renal tubules · IS Inflammation/Necrosis
BM Micronucleus	-	-	

- = No findings
= Parameter not evaluated due to early termination
Li = liver, Ki = kidney, Ht = heart, and IS = injection site

At termination, the high-dose animals grossly demonstrated marked tail vein irritation. This was associated with decreased body weight and a histopathology finding of injection site inflammation and venous necrosis. Liver and kidney weights were also increased in these animals. There were no such findings in the mid- and low-dose animals. The bone marrow micronucleus test was negative in the low- and mid-dose animals (bone marrow micronucleus was not evaluated in the high-dose group due to the early termination). The only other finding was the histopathologic observation of slight vacuolization of the renal tubule observed in the high-dose animals.

Conclusion

Although the study reported in Example 2 demonstrated that formulation with SBE-CD ameliorated the adverse effects of injection of pseudomycin B, adverse effects were observed with an SBE-CD formulation in the present study. However, the dose amount and concentration of pseudomycin B are significantly higher in the present study.

This study evaluated doses of pseudomycin B up to 50 mg/kg, which is twice the dose tested in the studies reported in Example 2, which was 25 mg/kg. Furthermore, the present study injected this doubled dose of pseudomycin B in a volume only one half that used in the studies reported in Example 2. Thus, the concentration of pseudomycin B in the formulation increased 4-fold. The concentration of SBE-CD remained constant at 2 wt-% in these studies, so the concentration of pseudomycin B increased 4-fold relative to the concentration of SBE-CD.

This study indicates that amelioration of adverse effects of injection requires at least a certain minimum concentration of SBE-CD relative to the concentration of pseudomycin B. Although not limiting to the present invention, this observation is consistent with formation of a complex of SBE-CD and pseudomycin B.

Example 5 -- Increasing the Concentration of SBE-CD at a High Dose of Pseudomycin B Ameliorates Adverse Effects

This study investigated toxicities of high dose pseudomycin B with an increased amount of SBE-CD and determined that higher concentrations of the cyclodextrin ameliorated the adverse effects of high dose pseudomycin B seen in Example 4.

Materials and Methods

Animals were as described in Example 4. The doses of pseudomycin B used for this study were 0, 25, or 50 mg/kg/day administered for 14 days as an intravenous slow injection using a computer controlled pump to minimize test article extravasation into surrounding tissue. The vehicle used for this study was 4 wt-% SBE-CD and the dose volume was 10 mL/kg. These increases in the concentration of SBE-CD and the dose volume this adjusted the ratio of pseudomycin B to SBE-CD back to what it was in the studies reported in Example 3. The control group received 4 wt-% SBE-CD alone. Body weights, clinical observations, mortality, hematology, clinical chemistry, limited pathology (liver, kidney, heart, and injection site), and bone marrow micronucleus were monitored by standard methods.

Results

The live phase of this study was completed with no evidence of overt toxicity, as is shown by the results reported in Table 6.

Table 6 -- Results of Administering 4 wt-% SBE-CD Formulation with High Concentrations of Pseudomycin B.

PARAMETERS	25 mg/kg/day	50 mg/kg/day
Clinical Observations	-	-
Mortality	-	-
Body Weight	-	-
Gross Pathology	-	-
Organ Weights (Li, Ki, and Ht)	· ↑Ki weight (9%)	· ↑Ki weight (14%)
Hematology	-	· decreased erythron values (slight anemia)
Clinical Chemistry	-	· ↑ALT & AST (1.5X) · ↓cholesterol · ↓tot protein/albumin · ↑BUN (very minor)
Histopathology (Li, Ki, Ht, and IS)	· Slight vacuolization of renal tubules	· Minor hepatocellular degeneration · Slight vacuolization of renal tubules
BM Micronucleus	-	-

- = No findings
[redacted] = Parameter not evaluated due to early termination
Li = liver, Ki = kidney, Ht = heart, and IS = injection site

Conclusion

Increasing the ratio of SBE-CD to pseudomycin B in the formulation completely ameliorated the irritation seen with the 50 mg/kg/day dose group in the study reported in Example 4. This is consistent with a mechanism of the cyclodextrin-mediated protection involving formation of a complex including pseudomycin B and the cyclodextrin, which results in protection of the vascular endothelium.

Amelioration of the adverse effects at the site of injection allowed the first observation of apparent target organ effects at the highest tested dose of pseudomycin B. The hematology, clinical chemistry, and histopathology data indicate target organ effects associated with pseudomycin B administration. The development of a slight anemia suggests effects on red blood cells or red blood cell producing elements of the bone marrow. The liver also emerged as a potential target organ with evidence of liver enzyme

leakage and hepatocellular injury and death. Both of these effects were seen only at the highest dose of 50 mg/kg/day. Increased kidney weight and slight vacuolization of the renal tubules was seen at both 25 and 50 mg/kg/day and may indicate renal effects of pseudomycin B.

Example 6 -- Evaluation of the Metabolism of Pseudomycin B Following Intravenous Injection of a SBE-CD Formulation

This study evaluated metabolism of a formulation of pseudomycin B with sulfobutylether-β-cyclodextrin following intravenous injection in rats.

Materials and Method

The doses of pseudomycin B used for this rat study were 5 and 25 mg/kg administered once as an intravenous bolus dose. The SBE-CD formulation was 2 wt-% and the dose volume used was 5 mL/kg. Parameters monitored by standard methods included area under the curve for the duration of detectable levels (AUC), half-life, and C_{max}.

Results

The results of this study are reported in Table 7.

Table 7 -- Metabolism Studies of Formulations of Pseudomycin B With SBE-CD.

Dose of PSB (mg/kg)	Formulation	AUC_0 (ng • hr/mL	Half-life (hours)	C_{max} (μg/mL)
5	NaOAc buffer	48,799	4.2	17.0
5	2 wt-% SBE-CD	41,156	4.0	20.6
25	2 wt-% SBE-CD	60,873	4.0	85.9

Conclusions

The plasma concentrations of pseudomycin B were similar after a 5 mg/kg dose in either sodium acetate buffer or in a formulation with 2 wt-% SBE-CD. The presence of 2

wt-% SBE-CD did not affect the plasma pharmacokinetic profile of pseudomycin B in comparison with acetate buffer. The formulation did not change any of the measured pharmacokinetic parameters. In the 2 wt-% SBE-CD formulation, increasing the dose of pseudomycin B from 5 to 25 mg/kg resulted in a near proportional increase in C_{max} and AUC. The plasma half-life of pseudomycin B was independent of dose examined. Measurable plasma concentrations of pseudomycin B were present for 24 hours after the administration of dose.

Example 7 -- *In vivo* Toxicological Evaluation of an HP-CD and a Gamma-Cyclodextrin Formulation of Pseudomycin B

This study further evaluated the ability of the hydroxypropyl-β-cyclodextrin and gamma-cyclodextrin formulations to provide protection from adverse effects intravenous injection of pseudomycin B.

Materials and Methods

Animals were male rats as described in Example 4 which were divided into four groups of five. The doses of pseudomycin B used for this study were 0 and 50 mg/kg/day for 14 days administered as an intravenous bolus in a dose volume of 10 mL/kg at a rate of 66 mL/hr. Hydroxypropyl-β-cyclodextrin was obtained from Cyclodextrin Technology Inc. Gamma-cyclodextrin was obtained from Wacker. The cyclodextrin concentration used in the vehicle preparations was 4 wt-% in pH 4.5 acetate buffer (0.05M). Control groups received either 4 wt-% gamma-CD or 4 wt-% hydroxypropyl-CD lacking pseudomycin B.

Results

The results of this study are reported in Table 8.

Table 8 -- Results of Administering Hydroxypropyl-β-Cyclodextrin and Gamma-Cyclodextrin Formulations at Two Concentrations of Pseudomycin B.

PARAMETERS	Hydroxypropyl-B-CD	Gamma-CD
Clinical Observations	• 2 animals demonstrated slight tail vein irritation at the end of the 2-week dosing period	• Dosing terminated on study day 2 due to excessive tail vein irritation
Mortality		
Body Weight	• Slight decrease in BW parameters (i.e., ↓BW (3.2%) at d13)	• Decrease in BW parameters (i.e., ↓BW (14.8%) at d6)
Gross Pathology	• ↑Kidney wt., absolute (8%) relative (11%)	
Hematology	• Changes consistent with a mild anemia	
Clinical Chemistry	• ↑Chol (~30%) and ↓Tri (~30%) • ↑ALT and AST (15-22% NSS) • ↑BUN (16%) • ↓Serum albumin (7%)	
Histopathology (Li, Ki, Ht, and IS)	• Renal cortical vacuolization in control (grade slight) and treated (grade moderate) animals • Perivasculitis occurred at the injection site of control (grade minimal) and treated (grade slight-moderate) animals. Inflammation of the muscle and vascular necrosis were found only in treated rats.	

Shading indicates no effect on the indicated parameter
Shading indicates the indicated parameter was not measured due to early termination

The gamma-CD vehicle did not provide adequate protection from adverse effects of pseudomycin B in this study. After only the first dose swelling and discoloration of the tail was noted. This became severe enough to prevent dosing by day 2.

The hydroxypropyl-β-CD vehicle provided protection from adverse effects of pseudomycin B. As a result, daily doses of the hydroxypropyl-β-CD formulation were administered for the full 2-weeks of the study. Adverse effects on the erythron component, liver, kidney, and lipid metabolism are consistent with previous observations following 2 weeks of dosing at the 50 mg/kg level using a SBE-CD-based vehicle (Example 5). There was some evidence of slight adverse effect at the site of injection at the gross and microscopic level in the present study. This was not reported in the previous SBE-CD vehicle study, suggesting that the SBE-CD, under the conditions tested, may provide a greater degree of protection than hydroxypropyl-β-CD vehicle.

Conclusion

In this study, gamma-CD did not provide protection from the adverse effects of injection of pseudomycin B. Hydroxypropyl-β-CD protected these animals from the adverse effects of injection of pseudomycin B.

The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

All publications and patent applications in this specification are indicative of the level of ordinary skill in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated by reference.

WE CLAIM:

1. A method of reducing the symptoms of a fungal infection in a patient in need thereof comprising:

administering to the patient an effective amount of a composition comprising a pseudomycin or a lipodepsidecapeptide antifungal agent, or a pharmaceutically acceptable salt, hydrate or ester thereof, and a hydroxyalkyl-β-cyclodextrin or sulfoalkylether-β-cyclodextrin.

2. The method of claim 1, wherein reducing the symptoms comprises decreasing a burden of a fungal infection.

3. The method of claim 1, wherein reducing the symptoms comprises killing the fungus.

4. The method of claim 1, wherein the wherein the fungal infection comprises infection by *C. parapsilosis, C. albicans, C. glabrata, C. tropicalis, C. krusei, Cryptococcus neoformans, Aspergillus fumigatus,* or *Histoplasma capsulatum.*

5. The method of claim 1, wherein the composition comprises hydroxypropyl-β-cyclodextrin.

6. The method of claim 1, wherein the composition comprises sulfobutylether-β-cyclodextrin.

7. The method of claim 1, wherein the patient suffers no unacceptable adverse effects of injection of the pseudomycin or the lipodepsidecapeptide antifungal agent, or a pharmaceutically acceptable salt, hydrate or ester thereof.

8. The method of claim 1, wherein a toxicologically relevant dose of the pseudomycin or the lipodepsidecapeptide antifungal agent, or a pharmaceutically acceptable salt, hydrate or ester thereof, is administered.

9. The method of claim 1, wherein the composition comprises the cyclodextrin in about a molar ratio of at least about 1 compared to the pseudomycin or the lipodepsidecapeptide antifungal agent administered.

10. The method of claim 1, further comprising:

determining the need for administering the pseudomycin or the lipodepsidecapeptide antifungal agent; and

monitoring the patient for signs of extravasation of the pseudomycin or the lipodepsidecapeptide antifungal agent.

11. The method of claim 1, wherein administering comprises parenteral administration about 1 to about 3 times per day of about 0.1 to about 5 mg/kg of the pseudomycin or the lipodepsidecapeptide antifungal agent.

12. The method of claim 1, wherein reducing the symptoms of a fungal infection comprises reducing fever and increasing general well being of the patient.

13. The method of claim 1, wherein the effective amount of the pseudomycin or the lipodepsidecapeptide antifungal agent is an effective antifungal amount.

14. The method of claim 1, wherein the pseudomycin comprises pseudomycin A, pseudomycin A', pseudomycin B, pseudomycin B', pseudomycin C, pseudomycin C', a combination thereof, or a pharmaceutically acceptable salt, hydrate or ester thereof.

15. The method of claim 14, wherein the pseudomycin comprises pseudomycin B or a pharmaceutically acceptable salt, hydrate or ester thereof.

16. The method of claim 1, wherein the lipodepsidecapeptide antifungal agent comprises 25-B1 decapeptide antifungal agent A, or a pharmaceutically acceptable salt, hydrate or ester thereof.

17. An antifungal composition comprising hydroxypropyl-β-cyclodextrin or sulfobutylether-β-cyclodextrin and an effective antifungal amount of a pseudomycin, a lipodepsidecapeptide antifungal agent, or a pharmaceutically acceptable salt, hydrate, or ester thereof; the hydroxypropyl-β-cyclodextrin or sulfobutylether-β-cyclodextrin being in a molar excess over the pseudomycin, the lipodepsidecapeptide antifungal agent, or pharmaceutically acceptable salt, hydrate, or ester thereof.

18. The antifungal composition of claim 17, comprising hydroxypropyl-β-cyclodextrin.

19. The antifungal composition of claim 17, comprising sulfobutylether-β-cyclodextrin

20. The antifungal composition of claim 17, wherein the composition comprises hydroxypropyl-β-cyclodextrin or sulfobutylether-β-cyclodextrin in at least about a molar ratio of 2:1 compared to pseudomycin or the lipodepsidecapeptide antifungal agent.

21. The antifungal composition of claim 17, further comprising dextrose and acetate buffer.

22. The antifungal composition of claim 17, wherein the pseudomycin comprises pseudomycin A, pseudomycin A', pseudomycin B, pseudomycin B', pseudomycin C, pseudomycin C', a combination thereof, or a pharmaceutically acceptable salt, hydrate or ester thereof.

23. The antifungal composition of claim 23, wherein the pseudomycin comprises pseudomycin B or a pharmaceutically acceptable salt, hydrate or ester thereof.

24. The antifungal composition of claim 17, wherein the lipodepsidecapeptide antifungal agent comprises 25-B1 decapeptide antifungal agent A or a pharmaceutically acceptable salt, hydrate or ester thereof.

25. Use of a pseudomycin or a lipodepsidecapeptide antifungal agent, or a pharmaceutically acceptable salt, hydrate or ester thereof, in combination with a hydroxyalkyl-β-cyclodextrin or a sulfoalkylether-β-cyclodextrin for the manufacture of a medicament for the treatment of a fungal infection.

26. The use of Claim 25 wherein the pseudomycin comprises pseudomycin A, pseudomycin A', pseudomycin B, pseudomycin B', pseudomycin C, pseudomycin C', a combination thereof, or a pharmaceutically acceptable salt, hydrate or ester thereof.

27. The use of Claim 25 wherein the lipodepsidecapeptide antifungal agent comprises 25-B1 decapeptide antifungal agent A or a pharmaceutically acceptable salt, hydrate or ester thereof.

28. The use of Claim 25 wherein the hydroxypropyl-β-cyclodextrin or sulfobutylether-β-cyclodextrin is present in the medicament in at least a molar ratio of 2:1 compared to the pseudomycin or the lipodepsidecapeptide antifungal agent.

SEQUENCE LISTING

<110> Eli Lilly and Company

<120> Pseudomycin Antifungal Compositions and Methods for Their Use

<130> X-11463 Sequence Listing

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